Mol Cell Biochem (2010) 340:239–247
DOI 10.1007/s11010-010-0423-8

Transient anoxia and oxyradicals induce a region-specific activation of MAPKs in the embryonic heart

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Received: 26 August 2009 / Accepted: 26 February 2010 / Published online: 21 March 2010

Abstract We have previously reported in the early septating embryonic heart that electromechanical disturbances induced by anoxia-reoxygenation are distinct in atria, ventricle, and outflow tract, and are attenuated in ventricle by opening of mitochondrial K_{ATP} (mitoK_{ATP}) channels. Here, we assessed the regional activation of mitogen-activated protein kinases (MAPKs) ERK, p38, and JNK in response to anoxia-reoxygenation and H_2O_2. Hearts isolated from 4-day-old chick embryos were subjected to 30-min anoxia and 60-min reoxygenation or exposed to H_2O_2 (50 μM–1 mM). The temporal pattern of activation of ERK, p38, and JNK in atria, ventricle, and outflow tract was determined using immunoblotting and/or kinase assay. The effect of the mitoK_{ATP} channel opener diazoxide (50 μM) on JNK phosphorylation was also analyzed. Under basal conditions, total ERK and JNK were homogeneously distributed within the heart, whereas total p38 was the lowest in outflow tract. The phosphorylated/total form ratio of each MAPK was similar in all regions. Phosphorylation of ERK increased in atria and ventricle at the end of reoxygenation without change in outflow tract. Phosphorylation of p38 was augmented by anoxia in the three regions, and returned to basal level at the end of reoxygenation except in the outflow tract. JNK activity was not altered by anoxia-reoxygenation in atria and outflow tract. In ventricle, however, the diazoxide-inhibitable peak of JNK activity known to occur during reoxygenation was not accompanied by a change in phosphorylation level. H_2O_2 over 500 μM impaired cardiac function, phosphorylated ERK in all the regions and p38 in atria and outflow tract, but did not affect JNK phosphorylation. At a critical stage of early cardiogenesis, anoxia, reoxygenation, exogenous H_2O_2 and opening of mitoK_{ATP} channels can subtly modulate ERK, p38, and JNK pathways in a region-specific manner.

Keywords p38 MAP kinase · ERK · JNK · Anoxia-reoxygenation · Embryonic heart · Oxyradicals

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Introduction

The embryonic/fetal heart develops and operates normally in a relatively hypoxic intrauterine environment, but reacts rapidly to oxygen lack [1–3]. Oxygen deprivation during critical periods of embryogenesis impairs heart development and function, resulting in growth retardation and increasing the risk of cardiovascular disease in adulthood [4–6]. Maternal hypoxemia, reduction in umbilical blood flow or placental dysfunction can rapidly lead to acute or chronic ischemia and/or hypoxia. Although the post-ischemic fetal heart seems to recover faster than the adult heart [7, 8], the tolerance of the embryonic heart to hypoxia and its capacity to recover during reoxygenation remain under debate. Moreover, the functional and developmental consequences of oxygen lack may vary from one cardiac region to another since the tissue properties and the fate of each part of the heart are different. Atria differentiate at older stages into pacemaker tissue, ventricle into working myocardium and outflow tract into aorta and pulmonary artery. In previous works, we have precisely characterized the electrical and contractile disturbances induced by

anoxia and reoxygenation in the heart isolated from 4-day-old chick embryos [9, 10]. Arrhythmias and myocardial stunning observed at reoxygenation are associated with a burst of reactive oxygen species (ROS) [9], and recovery of excitation–contraction coupling in the ventricle is improved by pharmacological activation of the mitochondrial K_{ATP} (mitoK_{ATP}) channels, via PKC, NO-, and ROS-dependent mechanisms. However, the signaling pathways underlying the response to anoxia-reoxygenation remain unclear in the embryonic heart.

Mitogen-activated protein kinases (MAPKs), which belong to a highly conserved family of serine/threonine kinases, are present in all eukaryotic cells and are signaling proteins that play a key role in response to a wide range of stress [11]. The three best-characterized MAPKs, extracellular signal-regulated protein kinase (ERK), c-jun NH_2-terminal kinase (JNK), and p38 MAPK have been involved in a vast array of physiopathological mechanisms in cardiac cells [12–15], and are notably implicated in ischemia–reperfusion injury and in pre- and post-conditioning mechanisms [16–19]. Previous studies, mainly focused on newborn or adult models [12], have reported conflicting data, and the involvement of MAPKs pathways in the response of the fetal heart to limiting oxygen levels has been poorly investigated. We have recently shown in the ventricle of the embryonic chick heart that JNK pathway is involved in the response to anoxia-reoxygenation and that reoxygenation-induced peak of JNK activity was ROS-independent and tightly related to the open-state of the mitoK_{ATP} channel [20]. Regarding the response to oxygen deprivation and reoxygenation, some differential sensitivity is expected within the embryonic heart since anoxic tolerance [9], energy metabolism [21], Ca^{2+} handling [22, 23], myofilaments [24], oxidative stress [25], sensitivity to NO [26], and electrical [27] and contractile [10] properties vary from one cardiac region to another. These important developmental, structural, and functional differences combined with the fact that activation of MAPKs depends on the nature of the stimuli and the cell-type [28] lead us to hypothesize that ERK, p38, and JNK pathways display distinct spatio-temporal patterns of activation in response to a transient anoxic stress. The main goal of the present work was to investigate the expression and the profile of activation of ERK, p38, and JNK in atria, ventricle and outflow tract of the embryonic heart submitted to anoxia, reoxygenation, and oxidant stress.

Methods

Reagents

All standard chemicals, as well as dimethylsulfoxide (DMSO), hydrogen peroxide (H_2O_2), and mitoK_{ATP} channel opener diazoxide were analytical grade and purchased from Sigma-Aldrich. [γ-^{33}P] ATP was from Amersham Biosciences and inhibitors of proteases from Roche Biosciences. Rabbit antibodies against phosphorylated-ERK1/2, phosphorylated-p38, phosphorylated-JNK1/2, total-ERK1/2, total-p38, total-JNK1/2 were purchased from Cell Signaling Technology. The antibody against α-actin was from Sigma-Aldrich and the secondary antibody (goat anti-rabbit HRP conjugated) was from GE Healthcare. The enhanced chemiluminescence (ECL) western blot reagent kit was from Pierce and the films from GE Healthcare.

Preparation and in vitro mounting of the heart

Because of the minute size of the heart (circa 60 µg proteins) and its parts at the stage investigated, a total of about 1000 chick embryos were utilized in this study. All experiments were performed in accordance with the guidelines of the local veterinary authority. Fertilized eggs from Lohman Brown hens were incubated during 96 h at 38°C and 95% relative humidity to obtain stage 24 HH embryo (according to Hamburger and Hamilton [29]). The spontaneously beating hearts were carefully excised from explanted embryos by section at the level of the truncus arteriosus as well as between the sinus venosus and the atria. The hearts were then placed in the culture compartment of an airtight chamber.

The stainless steel chamber was equipped with two windows for observation and maintained under controlled conditions on the thermostabilized stage (37.5°C) of an inverted microscope (IMT2 Olympus, Tokyo, Japan) as previously detailed [10]. Briefly, the culture compartment (300 µl) was separated from the gas compartment by a 15 µm transparent and gas-permeable silicone membrane (RTV 141, Rhône-Poulenc, Lyon, France). Thus, pO_2 at the tissue level could be strictly controlled and rapidly modified (within less than 5 s) by flushing high-grade gas of selected composition through the gas compartment. At this developmental stage, the heart lacks vascularization and the myocardial oxygen requirement is met exclusively by diffusion.

The culture medium was standard HCO_3/CO_2 buffered Tyrode medium, equilibrated in the chamber with 2.31% CO_2 in air (normoxia and reoxygenation) or in N_2 (anoxia) yielding a pH of 7.4. Diazoxide was diluted in this medium containing 0.5% DMSO (vehicle) and present throughout the experimental protocol.

Anoxia-reoxygenation protocol

After a 30 min preincubation at room temperature in vehicle or in diazoxide (50 µM), hearts were mounted in vitro and stabilized 45 min under normoxia at 37°C, and then submitted to strict anoxia during 30 min, followed by

60 min of reoxygenation. The hearts were harvested after the period of normoxic stabilization, after 30 min of anoxia and after 10, 30, and 60 min of reoxygenation. Atria, ventricle, and outflow tract of each heart were carefully dissected on ice and stored at -80°C for subsequent determinations.

Hydrogen peroxide (H_2O_2) exposure

The hearts were placed in a Petri dish in the standard medium, stabilized 45 min under normoxia and then exposed 1 h to 50, 100, 200, 500 or 1000 μM of H_2O_2 at 37°C. Cardiac rhythmicity and contractility were not different in hearts cultured in Petri dishes or in hearts mounted in culture chamber. The critical H_2O_2 concentration that impaired cardiac function was obtained by determining the proportion of hearts still beating at the end of each experiment. Atria, ventricle, and outflow tract were then carefully dissected on ice and stored at -80°C for subsequent determinations.

Protein extract preparation

Atria, ventricles, and outflow tracts were homogenized by sonication 3 × 2 s in the ice-cold lysis buffer. For each sample six atria, three ventricles, and six outflow tracts were pooled. Insoluble material was removed by 5 min centrifugation at 10,000×*g* and protein content was measured by the method of Bradford (Coomassie protein assay kit, Pierce) with bovine serum albumin as standard.

Immunoblotting

Proteins from whole cellular extracts (20 μg) were boiled with 33 vol.% of SDS sample buffer, separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes, which were probed with primary antibodies against MAPKs (1:1000) or against α-actine (1:5000) diluted in 5% bovine serum albumin in TBS-T (overnight, 4°C). The blots were then incubated (1 h, room temperature) with secondary antibody (1:10000) in 1% non-fat milk powder in TBS-T. Immunoreactive bands were detected with enhanced chemiluminescent procedure using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). The signal was semi-quantitatively analyzed using scanning densitometry (Quantity-One software, Biorad).

Protein bands were normalized to total-MAPKs or α-actin content in the same sample. In turn, the resulting densitometric ratio obtained during anoxia and reoxygenation was normalized to an internal control (the respective preanoxic ratio), and reported as fold increase. Thus, all the values >1 indicate a level of phosphorylation higher than the preanoxic level.

Kinase assay

JNK activity was determined according to a published method [30] with minor modifications [20]. Soluble protein extracts (30 μg) were incubated for 3 h at 4°C in the presence of 1 μg GST-c-Jun$_{(1–219)}$ bound to glutathione-agarose beads as both JNK-specific ligand and substrate. The beads were washed three times in washing buffer, and twice in kinase buffer. Kinase reaction was carried out for 30 min at 30°C in 20 μl of kinase buffer containing 5 μCi [γ-^{33}P]ATP. Reaction products were resolved by 12% SDS-polyacrylamide gel electrophoresis, gels were dried, and phosphorylation signals were visualized by autoradiography and quantitated by PhosphoImager (Quantity-One 1.4.0, Biorad) and normalized to the respective preanoxic control level.

Statistical analysis

Results are given as mean ± standard error of the mean (SEM). Statistical analysis was performed using Statistica 8.0 Software. Differences between time-points were determined by Kruskal-Wallis test followed by multiple comparisons post-hoc test. The significance of any difference between two conditions was assessed by Mann–Whitney test. The statistical significance was defined by a value of $P \leq 0.05$.

Results

Inhomogeneous MAPKs distribution under basal conditions

Under basal conditions (i.e., just dissected heart) the content of total form of ERK (p42) and JNK (p54) was similar in the three regions of the heart (Fig. 1a, b). However, in the outflow tract, the content of total form of p38 was lower than in atria and ventricle, and expression of p46 isoform of JNK was not detectable. In the three regions of embryonic heart p44 isoform of ERK was not expressed.

The phosphorylated to total form ratio of ERK, p38, and JNK was not significantly different in atria, ventricle, and outflow tract (Fig. 1b). Ventricle contained more α-actin than atria and outflow tract for the same quantity of protein (Fig. 1c).

Exogenous H_2O_2 phosphorylated MAPKs differently in atria, ventricle, and outflow tract

In order to determine to what extent myocardial MAPKs can be stimulated by H_2O_2, hearts were exposed to a concentration ranging from 50 μM to 1 mM. Cardiac

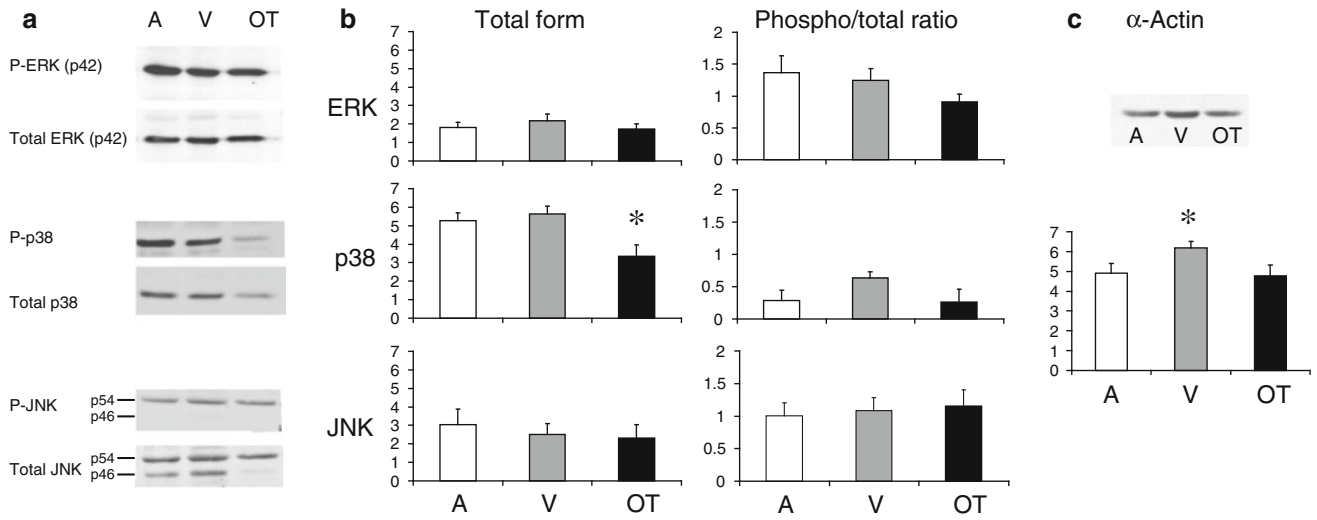

Fig. 1 Basal distribution of MAPKs and actin in atria (A), ventricle (V), and outflow tract (OT) of the embryonic chick heart at stage 24HH. **a** Representative immunoblots for phosphorylated and total ERK, p38, and JNK. **b** Densitometric analysis (arbitrary units) of total form and the phosphorylated to total form ratio. **c** Actin content. Only p38 (**a**, **b**) and JNKp46 (**a**) were inhomogeneously expressed. The phospho/total ratio was the lowest for p38 in the three regions. The content of α-actin was the highest in ventricle. The same quantity of protein was loaded in each lane of all the immunoblots (20 μg). $n = 4$–10 determinations; * $P < 0.05$ vs. A or V

function was clearly impaired by H_2O_2 at a rather high concentration (1 mM) which was subsequently used to assess its effects on the level of MAPK phosphorylation (Fig. 2a). Compared to controls, H_2O_2 promoted ERK phosphorylation in the whole heart, and significantly increased p38 phosphorylation only in atria and outflow tract. Surprisingly, the level of JNK phosphorylation was not altered by 1 mM H_2O_2, whatever the investigated region (Fig. 2b).

Anoxia-reoxygenation activated MAPKs differently in atria, ventricle, and outflow tract

The Fig. 3 shows the spatio-temporal modulation of the MAPKs in the embryonic heart subjected to anoxia and reoxygenation.

ERK phosphorylation

ERK phosphorylation increased during reoxygenation in atria and ventricle with a maximum reached at 60 min. In the outflow tract, reoxygenation did not alter significantly ERK phosphorylation.

p38 MAP kinase phosphorylation

After 30 min of anoxia, the level of p38 phosphorylation was about four-fold higher in the outflow tract than in atria and ventricle. During reoxygenation, the most important variations of p38 phosphorylation were observed in ventricle with a significant decrease at 60 min. A similar phenomenon was observed in the outflow tract but without statistical significance, most probably due to the large variability of the data. It should be noticed, however, that p38 remained activated in the outflow tract throughout reoxygenation, i.e., above its preanoxic level.

JNK activity versus phosphorylation

As determined by kinase assay, JNK activity increased in atria, ventricle, and outflow tract after 30 min of reoxygenation, but not significantly in atria and outflow tract (values reported for the ventricle are taken from our previous work [20]). Surprisingly, in contrast to activity, the level of JNK phosphorylation in the ventricle was not increased by reoxygenation (Fig. 4). Additionally, opening of the mitoK_{ATP} channels by diazoxide markedly reduced ventricular JNK activity during reoxygenation [20] but did not alter the level of phosphorylation (Fig. 4). These data show clearly that JNK activity did not parallel the level of JNK phosphorylation in the embryonic heart.

Discussion

To the best of our knowledge, this is the first time that distribution and activation of ERK, JNK, and p38 MAPK are explored in the three regions of an embryonic heart model submitted to a transient anoxic stress. Our main findings show that activation of MAPKs can be differentially modulated by anoxia-reoxygenation and by exogenous H_2O_2 in a region-specific manner.

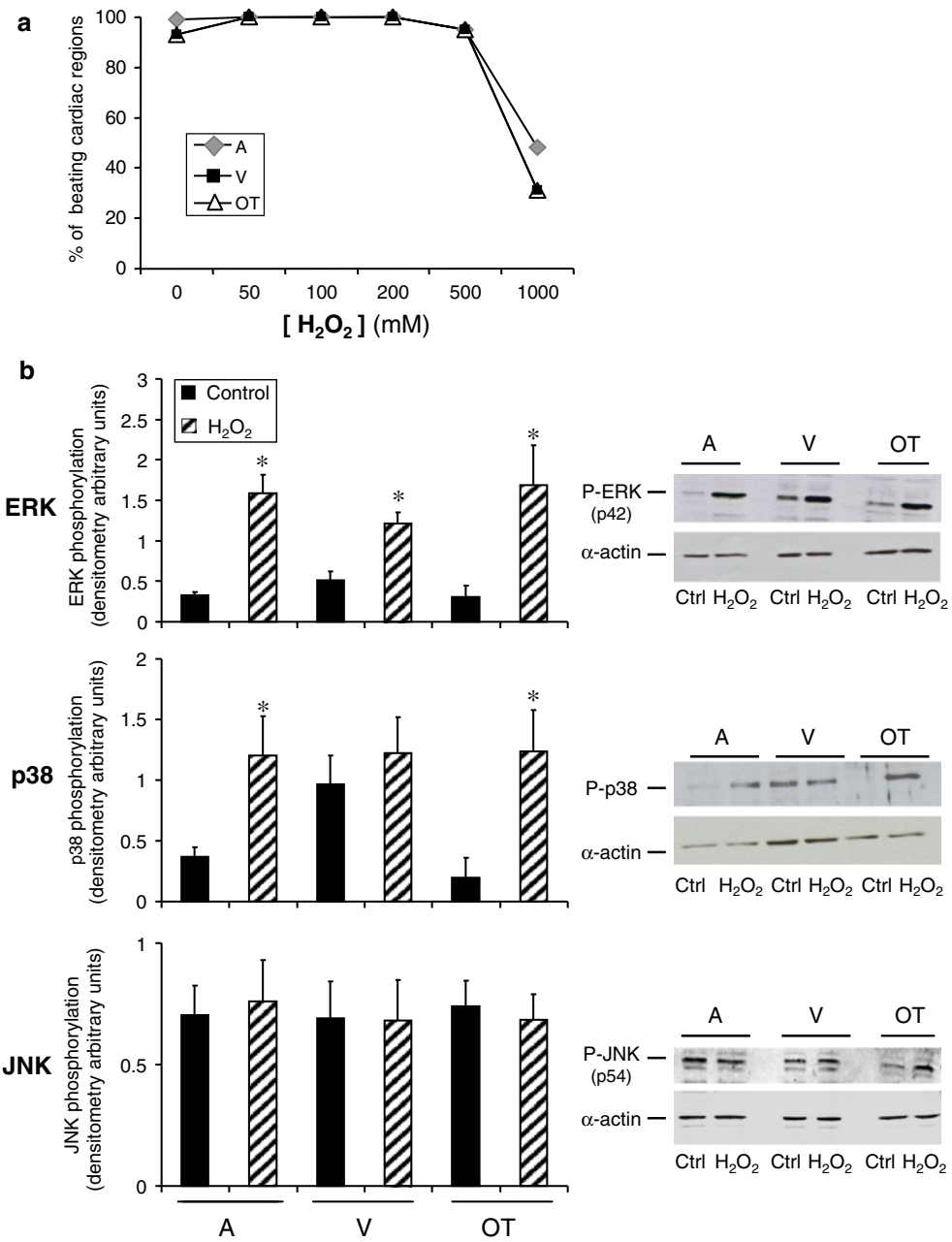

Fig. 2 Effects of H_2O_2 on contractile function and MAPKs phosphorylation. **a** A concentration of H_2O_2 higher than 500 μM was required to significantly affect contractions of A, V, and OT ($n = 100$ hearts). **b** H_2O_2 markedly increased phosphorylation of ERK and p38 specially in A and OT without modifying the level of JNK phosphorylation. Hearts were exposed to H_2O_2 1 h at 37°C. * $P < 0.05$ vs. control. $n = 5$ determinations

ERK and JNK were homogeneously distributed within the embryonic heart, whereas basal p38 expression was appreciably lower in the outflow tract relative to atria and ventricle (Fig. 1). It should be noticed that only the p42 isoform of ERK was detectable in the embryonic heart (Fig. 1a), alike in the adult chicken heart and different from neonatal and adult murine heart where both p42 and p44 isoforms were identifiable (not shown). Regarding p38 MAP kinase activation, the outflow tract appears also to be a unique part of the embryonic heart since it displayed both the lowest protein expression of p38 and the highest p38 responsiveness to anoxia and reoxygenation (Fig. 3). It should be mentioned that, at the stage investigated (24HH), the outflow tract undergoes important morphogenetic processes (remodeling) preparing the aorticopulmonary septation [31] which require an important physiological apoptotic activity [32, 33]. Indeed, in our preparation, there was 8.4 ± 1.3, 9.5 ± 0.3, and 14.5 ± 0.8% ($n = 3$ determinations) of apoptotic cells in atria, ventricle, and outflow tract, respectively (Pedretti and Yang, personal communication). Such a high level of apoptotic activity could partly explain that expression of p38 MAPK, which otherwise has been shown to display anti-apoptotic properties [12, 28], was the lowest in outflow tract. As expected, the highest relative amount of α-actin was found in the ventricle and was certainly due to the large number of proliferating and

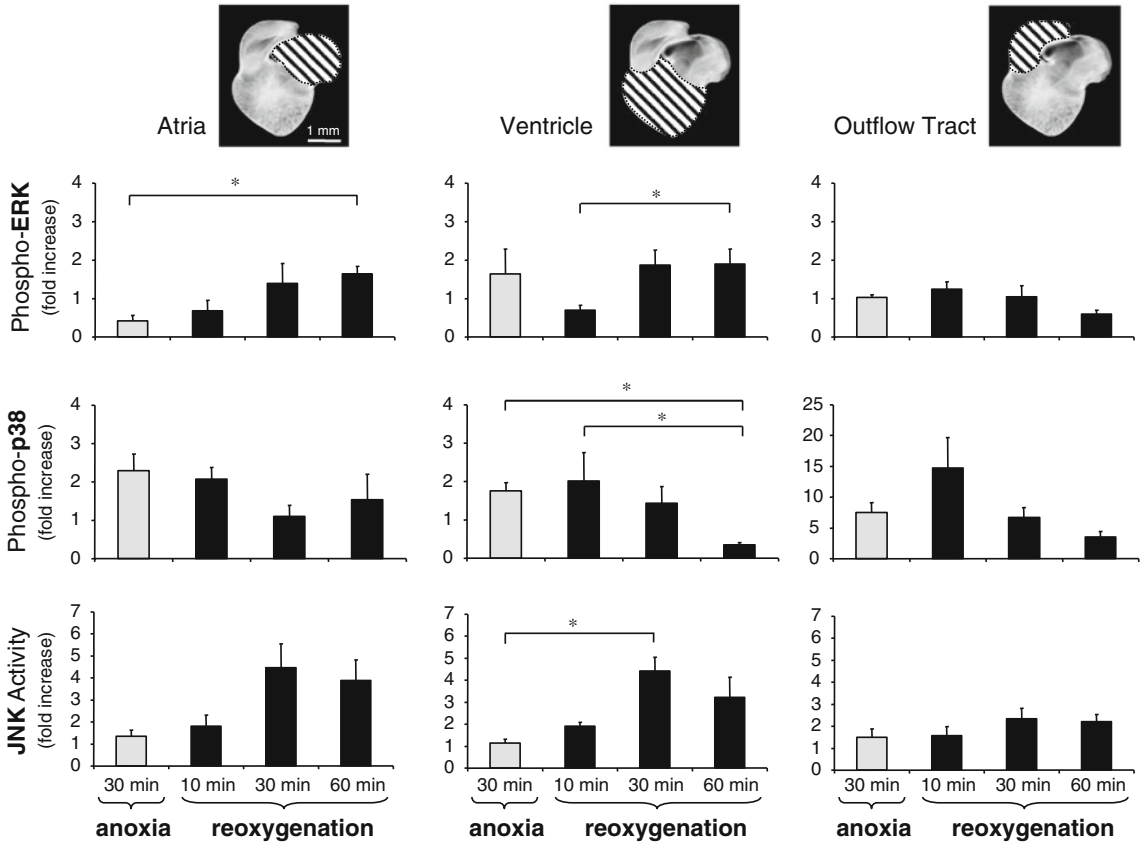

Fig. 3 Phosphorylation level of ERK and p38 MAPK and activity of JNK after 30 min of anoxia and 10, 30, and 60 min of reoxygenation in atria, ventricle, and outflow tract (hatched cardiac regions in *upper panel*). ERK, p38, and JNK displayed a characteristic profile of activation in each investigated region (see text). All values are expressed as fold increase relative to the preanoxic value obtained after 45 min of in vitro stabilization. * $P < 0.05$, $n = 3$–15 determinations for each column. Values reported for JNK activity in ventricle are taken from our previous work [20]

differentiating contractile myocytes in the developing compact and trabecular myocardial layers.

It is generally accepted that MAPKs are activated by ROS, especially by hydrogen peroxide (H_2O_2) [34, 35], although the degree and the time course of activation can differ according to the species, tissue, and cell-type [28, 36]. We sought to determine in atria, ventricle, and outflow tract to what extent exposure to a concentration of H_2O_2 sufficient to impair cardiac function, could also activate MAPKs. Under our experimental conditions the cardiac function was not altered by concentration of $H_2O_2 < 1$ mM whereas in fetal, newborn, and adult [37–39] cardiomyocytes contractions are already altered at 0.05 or 0.1 mM H_2O_2. Our data and these observations clearly indicate that the embryonic myocardium is less sensitive to this radical than the post-natal myocardium. This is corroborated by the fact that the functional recovery of the embryonic heart is rapidly completed after the reoxygenation-induced burst of ROS [9].

The most important effect of 1 mM H_2O_2 on ERK and p38 phosphorylation was observed in atria and outflow tract, which is consistent with the fact that 50% of atria stopped to beat at this concentration of H_2O_2 (Fig. 2a) and with the high sensitivity of the outflow tract to oxidative stress [40]. In the ventricle, the reoxygenation-induced peak of JNK activity is ROS-independent [20], and the present data show that JNK phosphorylation was not augmented by H_2O_2, whatever the region investigated (Fig. 2), demonstrating that JNK phosphorylation is not modulated even by the high oxidant stress induced by H_2O_2 exposure. These observations are in line with data obtained in adult myocardium showing that JNK is not activated by H_2O_2 at concentration as high as 500 μM [41] or by exposure to a pro-oxidant hyperoxia [42].

Relative to the other investigated MAPKs, ERK was modestly activated during anoxia and reoxygenation, suggesting that ERK is not a key element of the embryonic heart response to such a stress. ERK being not a stress kinase but mostly associated with cell proliferation and differentiation, it is not surprising that this pathway displayed such minor alteration under our experimental conditions. The low responsiveness of ERK to reoxygenation

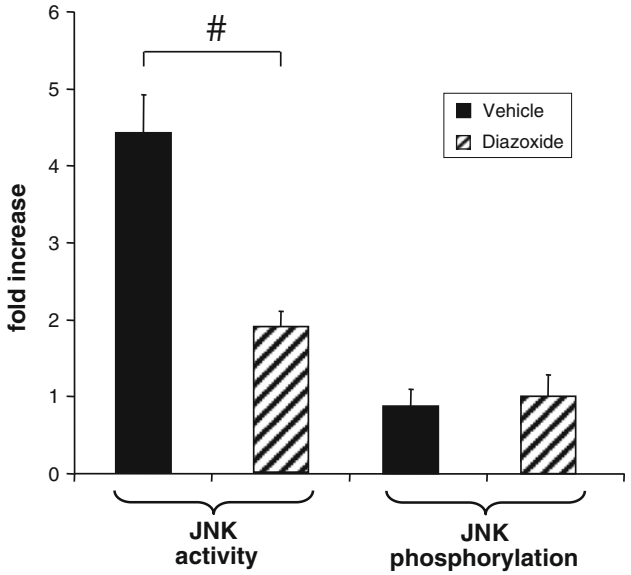

Fig. 4 After 30 min reoxygenation JNK phosphorylation did not parallel JNK activity in the ventricle. Reoxygenation strongly stimulated JNK activity but had no effect on JNK phosphorylation. Pharmacological opening of the mitoK$_{ATP}$ channel (diazoxide 50 μM) decreased the reoxygenation-induced JNK activity, but did not affect JNK phosphorylation. Values reported for JNK activity are taken from our previous work [20]. All values are expressed as fold increase relative to preanoxia value. $^{\#}$ $P < 0.05$ vs. vehicle; $n = 4$–10 determinations

suggests that the endogenous burst of ROS induced by reoxygenation is too short and/or too weak to strongly activate the kinase, in contrast to the severe exogenous oxidant stress generated by H_2O_2 which markedly increased ERK phosphorylation. It should also be noticed that ERK activity measured in the ventricle did not vary during anoxia-reoxygenation (Sarre and Maurer, personal communication).

Since the publication of Bogoyevith et al. [43] in 1996, several studies confirmed that p38 is strongly activated in heart by ischemia and involved in response to hypoxia and anoxia, but whether it displays a beneficial or a detrimental role remains under debate [44, 45]. In the present study, although the basal level of p38 expression was not homogeneous within the heart (Fig. 1b), anoxia increased phosphorylation in the three cardiac regions relative to preanoxic levels, especially in the outflow tract, (Fig. 3). Studies performed in adult [46, 47] and embryonic [9] cardiomyocytes showing that ROS production is totally abolished under strict anoxia, indicate that the observed anoxic increase of phospho-p38 was ROS-independent. The phosphorylation levels and kinase activities determined in this work displayed important interindividual variations which might be due to slight differences in developmental stage and/or to the relative sensitivity of the used techniques, including immunoblotting and kinase assay. Nevertheless, the greatest coefficient of variability of the level of p38 phosphorylation was consistently observed in ventricle and outflow tract after 10 min of reoxygenation. This suggests that during such a brief period of time, when ROS production is maximal, crucial cellular alterations may occur in these regions.

Although reoxygenation induces a calcium-dependent peak of JNK activity in the ventricle [20], we did not found the same pattern for JNK phosphorylation. Such a dissociation between JNK activity and phosphorylation could be partly related to the regulatory role played by scaffold proteins associated to JNK, e.g., JIP1, Sab, and Gst [48–50]. This important facet of JNK modulation is beyond the scope of our study. Likewise, an upward trend in JNK activity was observed in atria and outflow tract after 30–40 min reoxygenation, but without statistical significance.

We have previously shown that pharmacological opening of mitoK$_{ATP}$ channels not only reduces the reoxygenation-induced peak of JNK activity [20] but also improves ventricular recovery through a ROS-dependent mechanism [9]. However, in the present study, we found that diazoxide did not reduce JNK phosphorylation in ventricle, indicating that the modulation of JNK activity by the open-state of the mitoK$_{ATP}$ channels is not related to phosphorylation of the kinase, but rather to the level of intracellular calcium [20] and/or to alteration of JNK-interacting proteins (see above).

In conclusion, this study demonstrates for the first time that ERK, p38, and JNK show a characteristic distribution in the embryonic heart and are differentially modulated by anoxia-reoxygenation, exogenous oxyradicals, and mitochondrial K$_{ATP}$ channels in a region-specific manner. Even if the functional role of these MAP kinases remains to be clarified in such a model, our findings provide a first step in understanding the modulation of the signal transduction cascades in the developing heart subjected to oxygen lack and reoxygenation.

Acknowledgments The collaboration of Fabienne Maurer, Christophe Bonny, and Miguel Van Bemmelen, is gratefully acknowledged. We thank Anne-Catherine Thomas for her skillful technical assistance. This work was supported by the Swiss National Science Foundation n°3100A0-105901.

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